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Construction of the tissue engineering seed cell (HaCaT-EGF) and analysis of its biological characteristics

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ABSTRACT

Objective: To construct the tissue engineering seed cell (HaCaT cell line) with stable expression of the human epidermal growth factor (EGF), and analyze the changes of its biological characteristics. **Methods:** PCDNA3.1-EGF eukaryotic expression vector was transferred into HaCaT cell, and G418 was utilized to select the HaCaT-EGF cell line. Using an inverted microscope, PCR, ELISA method to detect the changes of the cell morphology, the expression of the EGF gene and protein, and the mRNA expression levels of apoptosis related molecule Caspase-3, the cell cycle related protein cyclin D1. **Results:** The mRNA expression levels of the obtained HaCaT-EGF cell were more than 100 times higher than the level of ordinary HaCaT cell. The colony of the HaCaT-EGF cells was more focused and tight compared to the empty vector transfected HaCaT cells and normal HaCaT cells. The expression levels of apoptotic factor Caspase-3 and cyclin D1 in HaCaT-EGF cell were significantly higher than those in the empty vector HaCaT- pcDNA3.1 cell, and the differences were statistically significant ($P<0.01$), but there was no significant difference compared to the normal HaCaT cells ($P>0.05$). **Conclusions:** HaCaT-EGF cell can continuously secrete EGF, and the biological characteristic is stable. It can be used for tissue engineering experiment and is an ideal seed cell for constructing tissue engineered skin.

1. Introduction

The development of tissue engineering skin provides a new train of thought for the repair and reconstruction of skin wound. At present, most seed cells of the tissue engineering skin come from epidermal cells and fibroblasts. However, for patients with large area burn, the epidermis damage is severe, the number of remaining epidermal cells is limited, the *in vitro* incubation time is long, and allogeneic cells are prone to produce rejection. Therefore, in the study on stem cells and tissue engineering skin, the optimization of seed cell source becomes a focus.

HaCaT cell is an immortalized cutin cell line isolated from adult skin, with the similar characteristics of proliferation, differentiation, stable genetic character and absence of tumorigenicity as cutin cells. Hence, it can be used as the seed cell candidates of tissue engineering skin, and is commonly used as the substitute in human keratinocyte experiment[1,2].

Human keratinocyte is an important cell for skin wound repair, and epidermal growth factor (EGF) is the key factor in the process of wound repair. EGF is mitogen between mesenchymal stem cells and epidermal cells, promoting cell proliferation, skin regeneration and angiogenesis, at the same time stimulating epidermic cells to synthesize secreted collagen, hyaluronic acid and other extracellular matrix, and promoting the growth of connective tissue cells[3]. EGF is a powerful chemotactic factor, which can promote epidermal cells, fibroblasts and endothelial cells to migrate to injury parts. In addition, as mitogen, EGF

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could promote cell proliferation, and thus has the function of promoting wound healing. There are researches[4] suggesting that human EGF plays an important role in the gland reconstruction. This research used EGF to transfect human keratinocyte line (HaCaT) successfully, obtained the HaCaT cells with stable and highly expressed EGF , and analyzed its influence on HaCaT biological characteristics, which can lay a solid foundation for building "complete tissue engineering skin" .

2. Materials and methods

2.1. Main reagents and equipments

Trypsin (Sigma Company, America), DMEM medium (Gibco Company, America), fetal calf serum (Gibco Company, America), TRIzol reagent (Invitrogen Company, America), reverse transcription reaction reagent kit, SYBR Green fluorescence quantitative reagent kit (TaKaRa Biotechnology Company, Japan), pcDNA3.1 (+). Lipofectamine™ 2000 (Invitrogen Company, America), human EGF Elisa kit, Caspase-3 Elisa kit and Cyclin D1 Elisa kit (Shanghai Saimo Biological Technology Co., Ltd, China). IX71 type inverted fluorescence microscope (Olympus Company, Japan), DU800 type nucleic acid protein analyzer (Beckman Coulter Company, America), IQ5TM fluorescence quantitative PCR instrument (Bio-Rad Company, America).

2.2. Cell line and plasmids

Keratinocyte (KC) was selected from HaCat cell lines of human keratinocytes (purchased from standard biological samples collection centers in the United States). The conventional recovery cultivation applies DMEM culture medium with volume fraction of 10% FBS to conduct, changing the medium 1 time every 2-3 d, subcultivation begins when the cell fusion reaches 90% under the condition of 37 ℃ and 5% CO_2 volume fraction. The pcDNA3.1-EGF recombinant plasmid was constructed and saved in our center.

2.3. Cell transfection and screening

HaCaT cell with good growth was selected. After digestion with 0.25% trypsin-0.1% EDTA, the cells were inoculated to 24-well culture plate with 2×10^4/hole. The pcDNA3.1-EGF plasmid and pcDNA3.1 empty plasmid were transfected into HaCaT cells respectively according to the specification of the liposome transfection reagent Lipofectamine™ 2000, and G48 working liquid was added after 24 h of transfection, the final concentration is of 600 μg/mL, the culture medium was changed 1 time every 3 d, and the non-transfected group was used as the negative control. Two weeks later the non-transfected HaCaT cells were all dead, transfection pcDNA3.1-EGF plasmid HaCaT cells, empty plasmid pcDNA3.1 showed clone growth, which were used to do limited dilution and subculturing in 96-wellplate. After cultured for 1 week, the monoclonal positive cells were picked and inoculated in the 24-well plate. When the cell fusion reached 70%-80%, cell was subcultured in 6-well plate and marked as HaCaT-EGF, HaCaT-pcDNA3.1 respectively.

2.4. HaCaT-EGF morphological observation

HaCaT, HaCaT EGF, HaCaT pcDNA3.1 cells were inoculated in the T25 culture bottle in accordance with the standard of 5×10^5/bottle. Culture medium was changed every 2-3 d. The cell morphology was observed with inverted microscope.

2.5. mRNA expression levels of EGF, caspase-3 and cyclin D1

HaCaT, HaCaT-EGF and HaCaT-pcDNA3.1 cells were all vaccinated in the 60 mm culture dish by the standard of 2.5×10^5. Cells in each group were collected when the cell fusion reached 80%. The totalRNA was extracted with Trizol reagent kit and cDNA was synthesized with reverse transcription kit. SYBR Green fluorescence quantitative kit was utilized for polymerase chain reaction with a total system of 20 μL, which contained 10 μL SYBRGreen mixture, 0.5 μL (10 μmol/L) Primer 1, 0.5 μL (10 μmol/L) Primer 2, 1 μL cDNA and 8 μL ddH_2O. Fluorescence quantitative PCR employed two-step and melting curve, whose amplification conditions were: 95 ℃, 30 s; 95 ℃, 10 s; 60 ℃, 20 s for 40 cycles, using GAPGH as internal parameters (primer GAPGH shown in Table 1). The iQ5™ fluorescent quantitative PCR instrument was adopted to analyze fluorescent quantitative PCR, repeated three times for each sample. The data was analyzed by analysis system of PCR instrument and relative gene expression analysis by $2^{-\triangle\triangle CT}$, with GAPDH as the internal parameter. Setting the expression of control group to 1, we calculate the relative expression of mRNA of experimental group EGF, caspase-3 and cyclin D1. The gene primers were shown in Table 1.

Table 1
Real-time fluorescent quantitative PCR primer sequence and product size.

Genes	Primer sequence	Product size (bp)
EGF	Forward: GAATGTCCCCTGTCCCACG	140
	Reverse: TCCCACCACTTCAGGTCTCG	
Caspase-3	Forward: GACTCTGGAATATCCCTGGACAACA	143
	Reverse: CTGAGGTTTGCTGCATCGACA	
Cyclin D1	Forward: ATGTTCGTGGCCTCTAAGATGA	138
	Reverse: CAGGTTCCACTTGAGCTTGTTC	
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC	138
	Reverse: TGGTGAAGACGCCAGTGGA	

2.6. Protein expressions of EGF, Caspase-3 and Cyclin D1 cells with ELISA method

HaCaT, HaCaT-EGF and HaCaT-pcDNA3.1 cells were inoculated in the 60 mm culture dish by the standard of 2.5 $\times 10^5$/bottle. The cell culture supernatant in each group was collected and saved at -80 ℃ when cell fusion reached 80%, and the EGF content in each cell culture supernatant was detected by ELISA. After cell collection and cell lysis with lysate containing PMSF, centrifuge was performed at 4 ℃, and supernatant was separated for ELISA test of Caspase-3 and Cyclin D1 protein.

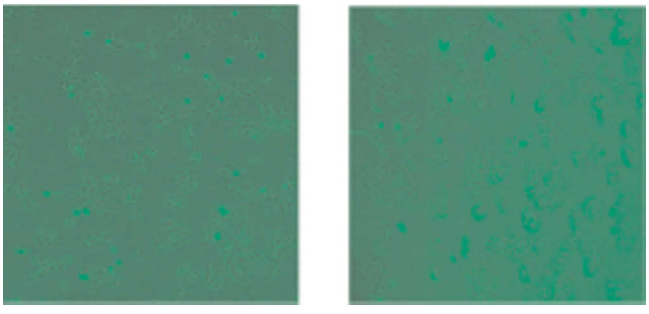
2.7. Statistical analysis

The experimental data were expressed by (mean±SD). One-way analysis of variance was performed for experiment results with the SPSS 17.0 statistical software; multiple comparisons were achieved using LSD-t, $P<0.05$ was of statistical significance.

3. Results

3.1. Cell screening and morphological observation of stable transfected HaCaT cell

HaCaT cell was processed with pcDNA3.1-EGF, pcDNA3.1empty vector plasmid and transfection reagent complex for 24 h. Then, cell screening was conducted by adding G418 solution. One week later, cells in transfection group had the cloned cell growth; obvious cell colony appeared 2 weeks later and the non-transfected cells were almost all dead. Then stable transfected cells could passage repeatedly in the condition of 200 μg/mL G418. Compared to the HaCaT cell with relatively loose cell colony, HaCaT-EGF cells thrived, cell colonies were more concentrated and compact (Figure 1).



A. HaCaT group(×100) B. HaCaT-pcDNA3.1-EGF group(×100)

Figure 1. Cell morphology observation.

3.2. mRNA expression levels of EGF, caspase-3 and cyclin D1

The total RNA in each cell was extracted, and total cDNA was acquired through reverse transcription. Fluorescent quantitative PCR was used to detect the gene mRNA level. Relative expression level of EGF mRNA in HaCaT-EGF group was 148.675±1.412, 100 times higher than that of HaCaT-pcDNA3.1 group (1.401±0.078) and HaCaT group (1.255±0.024). Relative expression level of Caspase-3 mRNA in HaCaT-EGF group was 1.273±0.019, lower than that of HaCaT-pcDNA3.1 group (2.628±0.151), the difference was of statistical significance (t=5.11, P=0.006), but there was no statistical difference compared with HaCaT group (1.000±0.579) (t=3.18, P=0.082). The mRNA relative expression level of Cyclin D1, HaCaT-EGF cell cycle-related protein, was 0.737±0.049, lower than the HaCaT-pcDNA3.1 group (2.167±0.070), with statistical significant difference (t=9.442, $P<0.01$); but there was no statistical significance difference compared to HaCaT group (1.000±0.052) (t=2.831, P=0.066) .

3.3. Protein expressions of EGF, Caspase-3 and Cyclin D1

The results of EGF protein expression by ELISA indicated that cells in HaCaT group (24.00±10.05) and HaCaT-pcDNA3.1 group (11.00±6.45) almost appeared no EGF protein expression, yet EGF expression in HaCaT-EGF group was (387.00±25.68) pg/mL. The result of Caspase-3 expression showed that the expression of Caspase-3 in HaCaT-EGF group was (201.00±15.56) pg/mL, and the difference was not significant ($P>0.05$) compared to HaCaT group [(197.00±17.33) pg/mL], but the difference was significant compared to HaCaT-pcDNA3.1 group [(394.00±12.03) pg/mL] ($P<0.01$). The results displayed that Cyclin D1 expression of HaCaT-EGF group was (158.00±14.21) pg/mL, the difference was not statistically significant ($P>0.05$) compared to HaCaT group (164.00±10.38) pg/mL, apparently lower than that of the HaCaT- pcDNA3.1 group (367.00±15.49) pg/mL ($P<0.05$).

4. Discussion

HaCaT cell line comes from normal human abdominal skin, immortalized cells formed by transfection cells of human papilloma virus. Though HaCaT cells have already shown their transfected cells phenotype, they had no signs of tumorigenicity and invasiveness[4]. HaCaT cell line was formed by induced human keratinocyte with the features of immortality and proliferation, differentiation similar to normal keratinocyte, therefore it was used as seed cells for

the *in vitro* culture of skin tissue engineering[5].

Skin is one of the ideal target organs of many gene therapies[6,7], keratinocyte and dermal fibroblast, being main component cells in the skin, have become primary cells of the current skin tissue engineering. The clinical study has demonstrated that EGF secretions increased in the early burn, but cell proliferation and granulation tissue with speedy hyperplasia leads to low content of EGF in blister fluid resulting in that burn wounds of EGF in the micro environment could not achieve the level of restoration, not meeting the maximum demand of wound repair. But exogenous EGF could quicken the healing significantly. Epidermal keratinocyte has the function of autocrine and paracrine which can secrete various cytokine, EGF gene expression vector can not only transform into human keratinocyte through *in vitro* culture and living way, but also participate in the regulation of adjacent cells such as fibroblasts with the help of exocrine mechanism[8,9]. Therefore, it is of great significance in the construction of tissue engineering skin and inducing the formation of complete engineering skin to select epidermal keratinocyte as the tool cell to acquire the capacity of sustained secretion of EGF.

In this experiment, we successfully screened an immortalized keratinocytes strain-HaCaT cell line which can secrete EGF. Cells of stable transfection could subculture repeatedly under the condition of 200 μg/mL G418, HaCaT-EGF cell colony were more concentrated and compacted compared to normal HaCaT cell and empty vector transfected HaCaT cell.

Caspase-3 was key cytotoxic effectors of cell apoptosis, it was found that relative expression of Caspase-3m RNA was lower than empty vector cell through HaCaT-EGF Caspase-3 expression detection, which was of no statistical difference. It had no effect on cell apoptosis after cell transfection indication and HaCaT-EGF cell could be cultured with continuous proliferation

Cyclin D1 was crucial regulatory factor of G_1 phase in cell cycle, cdk4/6-cyclin D1 compounds could phosphated RB protein, up-regulate the target gene expression of E2F to provide access for entering S phase by passing G1 phase checkpoint[10]. Down regulation of cyclin D1 could promote G_1/S phase transformation and shorten G_1 phase, accelerating cell entering S phase and cell proliferation[11]. We tested the results of HaCaT-EGF cyclin D1 expression, showing that the expression of HaCaT cyclin D1 declined sharply after introduction of EGF and quicken cell cycle progression to advance cell proliferation. The *in vitro* cultured HaCaT-EGF was more stable and more convenient to construct tissue engineering skin

The formation of EGF induced glands involved multiple signal molecules, HaCaT-EGF cells were used as tissue engineering seed cells to construct complete skin with other cells, which need further study in the subsequent experiments.

Conflict of interest statement

We declare that we have no conflict of interest.

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